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DISTRIBUTION AND INDUCTION OF CYP3A1 AND CYP3A2 IN RAT LIVER AND EXTRAHEPATIC TISSUES

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Abstract—Previously, we have shown that highly specific antibodies against cytochrome P450 enzymes can be produced by targeting a 5-amino acid sequence at the C-terminus. Although rat CYP3A1 and CYP3A2 share 89% amino acid sequence similarity, they differ by 3 out of 5 of their C-terminal residues. In an effort to produce antibodies specific to each form, rabbits were immunised with the peptides IITGS and VINGA, corresponding to the C-termini of CYP3A1 and CYP3A2, respectively. Both antibodies bound strongly to hepatic microsomal fraction from rats treated with pregnenolone 16α-carbonitrile (PCN) in enzyme-linked immunosorbent assay. Binding of the anti-IITGS antibody was strongly inhibited by incubation with IITGS, but VINGA was 60 times less effective. Conversely, binding of the anti-VINGA antibody was inhibited by VINGA 100 times more effectively than IITGS. Similar inhibition of antibody binding was also found using immunoblotting. Immunoadsorption using the anti-IITGS antibody yielded a single protein from solubilised hepatic microsomal fraction from PCN-treated rats, which was recognised only by the anti-IITGS antibody. Both antibodies bound to single proteins in the liver which were increased following treatment with PCN, but only the anti-IITGS antibody recognised protein in the lung, small intestine, and kidney of untreated and PCN-treated rats. Also, the binding of the two antibodies to hepatic and extrahepatic microsomal fractions from uninduced and induced rats showed differences in the expression of proteins recognised by the two antibodies, providing further evidence of antibody specificity. Thus, the binding of anti-IITGS and anti-VINGA antibodies is mutually exclusive and consistent with specific binding to their target antigens, CYP3A1 and CYP3A2, respectively. Immunocytochemistry was used to determine the distribution of CYP3A1 and CYP3A2. In the liver of untreated animals, both CYP3A1 and CYP3A2 were found to be expressed in the centrilobular region. However, some CYP3A1 immunoreactivity was also detected in many, but not all, hepatocytes throughout the lobule. However, following treatment of rats with PCN, both CYP3A1 and CYP3A2 were found to be strongly expressed in hepatocytes throughout the lobule, although CYP3A2 showed greater expression in the centrilobular region. PCN treatment was also found to result in induction of CYP3A1 in specific regions of the small intestine, lung, and kidney.

Key words: cytochrome P450; CYP3A1; CYP3A2; antipeptide antibodies; induction; pregnenolone 16α-carbonitrile.

P450† enzymes play a key role in the metabolism of a large number of both exogenous and endogenous substances [1]. This is accomplished by multiple forms of P450 with overlapping substrate specificities [2]. P450 enzymes are now recognised as a superfamily of haemoproteins with each member classified according to its primary structure [3].

P450 enzymes in the CYP3A subfamily catalyse the 6β-hydroxylation of testosterone [4] and the metabolism of a number of drugs including cyclosporin A [5], terfenadine [6], mephenytoin [7], nifedipine [8], and warfarin [9]. These P450 enzymes are inducible by glucocorticoids such as dexamethasone, macrolide antibiotics such as erythromycin, and PCN [10, 11]. In the rat, full-length cDNA clones encoding two PCN-inducible P450 enzymes have been isolated and sequenced [12, 13]. The proteins, which have subsequently been named CYP3A1 and CYP3A2 [3], share 89% amino acid sequence similarity.

To study the distribution and regulation of these two structurally very similar proteins, we decided to produce specific antipeptide antibodies against them. To do this, it was necessary to target a region that is substantially different in the two P450 enzymes, but the extensive sequence similarity between them meant there were few candidate regions. However, at the C-terminus 3 out of 5 amino acid residues vary between the two enzymes. We have previously shown that antibodies against P450 enzymes may be reliably produced by targeting a small region at the C-terminus [14]. Here, this approach was applied to the CYP3A subfamily in the rat. Thus, antibodies were raised against the C-terminal regions of both CYP3A1 and CYP3A2. The resulting antibodies were specific for the respective P450 enzyme and were used to study the distribution and regulation of these enzymes.

MATERIALS AND METHODS

Materials

N-α-9-Fluorenylmethoxycarbonyl protected amino acids linked to 4-benzyloxybenzyl alcohol resin, *N*-α-9-fluorenylmethoxycarbonyl amino acid pentafluorophenyl and 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine esters were purchased from Calbiochem-Novabiochem (Nottingham, U.K.) as was KLH. Acetonitrile (grade S) was from Rathburn Chemicals (Walkerburn,

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† Abbreviations: P450, cytochrome P450; PCN, pregnenolone 16α-carbonitrile; KLH, keyhole limpet haemocyanin; UT, untreated; MC, 3-methylcholanthrene; PB, sodium phenobarbitone; CFA, clofibric acid; STZ, streptozotocin.

U.K.). Polystyrene 96-well microtitre plates were from Dynatech Laboratories (Billinghurst, U.K.). All SDS-polyacrylamide gel electrophoresis reagents were from National Diagnostics (Aylesbury, U.K.). Hybond-C nitrocellulose hybridisation membrane, enhanced chemiluminescent reagents, and Hyperfilm were purchased from Amersham (Aylesbury, U.K.). Antirabbit immunoglobulin conjugated to horseradish peroxidase was from Sigma (Poole, U.K.). Biotinylated swine antirabbit immunoglobulin, StreptABComplex-horseradish peroxidase, and normal swine serum were from Dako (Patts, Denmark). Cyanogen bromide-activated Sepharose was from Pharmacia (St Albans, U.K.). All other chemicals were purchased from Sigma or Merck-BDH (Lutterworth, U.K.) and were of analytical grade or the best equivalent.

Treatment of animals, preparation of microsomal fractions

Male and female Wistar rats (200–250 g) were obtained from Harlan Olac Ltd (Bicester, U.K.). Groups of male rats were left UT or were administered PCN, MC, PB, CFA, or vehicle only by intraperitoneal injection as described by Rich *et al.* [15]. Groups of male rats were also treated intraperitoneally with STZ dissolved in 0.05 M sodium citrate buffer, pH 4.5 at a dose of 90 mg/kg; a second dose was administered 7 days later and the rats were killed after a further 7 days when they exhibited hyperglycaemia. In addition, groups of female rats were UT or treated with PCN, as described above. Rats were killed humanely in accordance with approved Home Office procedures. Liver, kidneys, lungs, and small intestine were rapidly removed and cooled in ice-cold 0.25 M potassium phosphate buffer, pH 7.25, containing 0.15 M potassium chloride. The small intestine was perfused with the same buffer that was supplemented with 5 mM dithiothreitol and 1 mM phenylmethylsulphonyl fluoride. The small intestine was cut laterally and the mucosal layer was removed and collected by scraping the tissue with a glass microscope slide. Microsomal fractions were prepared as described previously [16], except that 5 mM dithiothreitol and 1 mM phenylmethylsulphonyl fluoride were added to the homogenisation buffer when preparing microsomal fractions from lung and small intestinal mucosal cells.

Peptide synthesis, conjugation to carrier protein and production of antibodies

Synthesis of peptides, their conjugation to a carrier protein, KLH, and the immunisation of rabbits were as described previously [17]. The peptides synthesised were IITGS, VINGA, CIITGS, CVINGA, and the structurally unrelated FSAR and CFSAR. Cysteine was added to the N-terminus of peptides for conjugation to KLH as described previously [17]. A null conjugate (KLH-Cys) was produced by substituting cysteine for the thiolated peptide. All peptides were >95% pure as determined by reversed-phase high-pressure liquid chromatography under the conditions described previously [17]; they had the correct composition by amino acid analysis and the expected molecular weight determined by fast atom bombardment mass spectrometry. Male New Zealand white rabbits (3 kg) from Froxfield Farms Ltd. (Petersfield, U.K.) were immunised with peptides coupled to KLH as described previously [17].

ELISA

ELISA was performed as described previously [17].

Immunoblotting

Details of the method used have been described previously [17, 18]. Briefly, immunoblotting was performed using up to 30 μg of microsomal protein, as appropriate, and blots were developed with either anti-IITGS antiserum diluted 1:16000 or anti-VINGA antiserum diluted 1:4000. Binding of the antibodies was detected using goat antirabbit IgG coupled to horseradish peroxidase diluted 1:30000 and visualised using enhanced chemiluminescence, the result being recorded on Hyperfilm. The intensity of the bands was determined by laser densitometry using an LKB UltroScan XL Enhanced Laser Densitometer (Pharmacia LKB Biotechnology, St Albans, U.K.). Quantification studies were performed using appropriate dilutions of microsomal fractions to obtain a linear relationship between protein loading and band intensity.

Immunoadsorption

The IgG fraction of antiserum was purified using GammaBindG-Agarose. GammaBind G-Agarose gel (5 mL) was packed into a small chromatography column and washed with 0.1 M sodium phosphate buffer containing 0.15 M sodium chloride, pH 7.0 (loading buffer). Whole serum (5 mL) was mixed with 5 mL of loading buffer and passed through the column at a flow rate of 1 mL/min, followed by loading buffer until the UV absorbance of the eluent declined to zero. The IgG fraction was eluted in 0.5 M acetic acid, pH 3.0, neutralised immediately with 0.3 volumes of 3 M Tris-HCl, pH 8.8, and then dialysed against three changes of PBS. Purified IgG was coupled to CNBr-activated Sepharose at a ratio of 2 mg protein/mL gel as described previously [19]. IgG-Sepharose was suspended in PBS. Hepatic microsomal fraction from PCN treated rats was diluted to 2 mg/mL and then solublised by addition of sodium cholate to a final concentration of 0.6% (w/v) in 0.1 M phosphate buffer containing 20% (v/v) glycerol, 0.2 mM EDTA, pH 7.25 at 4°C. Solubilized protein (6 mg) was mixed with IgG-Sepharose (0.1 mL) overnight at 4°C. The IgG-Sepharose was washed with 0.1 M phosphate buffer containing 20% (v/v) glycerol, 0.2 mM EDTA, pH 7.25, and then 0.5 mL of 0.5 M Tris-HCl, pH 6.8, containing 20% (v/v) glycerol, 10% (w/v) SDS, and 0.5% (w/v) bromophenol blue was added and the mixture heated to 100°C for 3 min. IgG-Sepharose was removed by centrifugation at 500 g for 5 min, the supernatant was removed, 2-mercaptoethanol added to a final concentration of 1% (v/v), and the sample was subjected to immunoblotting as described above.

Immunocytochemistry

Samples of liver, kidney, small intestine, and lung tissue were fixed in 4% (v/v) formaldehyde overnight and then stored in 70% (v/v) ethanol. They were dehydrated in a graded series of ethanol solutions and then embedded in paraffin wax. Sections of approximately 3 μm thickness were cut on a microtome (Anglia Scientific, Cambridge, U.K.) onto poly-L-lysine coated slides and dried at 37°C. Paraffin wax was removed using a graduated series of solutions containing decreasing concentrations of ethanol. Sections were incubated for 30 min in distilled water containing 0.3% (v/v) hydrogen

peroxide to reduce endogenous peroxidase activity, and then in normal swine serum (1:20) to block nonspecific binding of antibodies. The following steps were performed in a humidified chamber. Sections were incubated for 2 hr at room temperature with antipeptide antibody (the optimal dilution was determined from serial dilutions). This was followed by a 1-hr incubation with biotinylated swine antirabbit IgG at a dilution of 1:500, and then streptavidin-biotin-peroxidase complex at a dilution of 1:500 as a third layer. Washing with PBS followed each incubation. Finally, peroxidase activity was revealed by immersion for 7 min in a solution of PBS containing 25 mg/mL 3,3′-diaminobenzidine tetrahydrochloride and 0.3% (v/v) hydrogen peroxide.

RESULTS

To determine if the antibodies bind to different antigens, their binding to hepatic microsomal fraction from PCN-treated rats was determined in competition with each of the peptides. There was a clear difference between IITGS and VINGA in competing with hepatic microsomal fraction for antibody binding. Binding of the antibody raised against IITGS (the C-terminus of CYP3A1) was strongly inhibited by IITGS, whereas VINGA was 60 times less effective (Fig. 1a). Conversely, binding of the antibody raised against VINGA (the C-terminus of CYP3A2) was strongly inhibited by VINGA, whereas IITGS was 100 times less effective (Fig. 1b). In comparison, the structurally unrelated peptide FSAR had little effect on the binding of either antibody (Fig. 1).

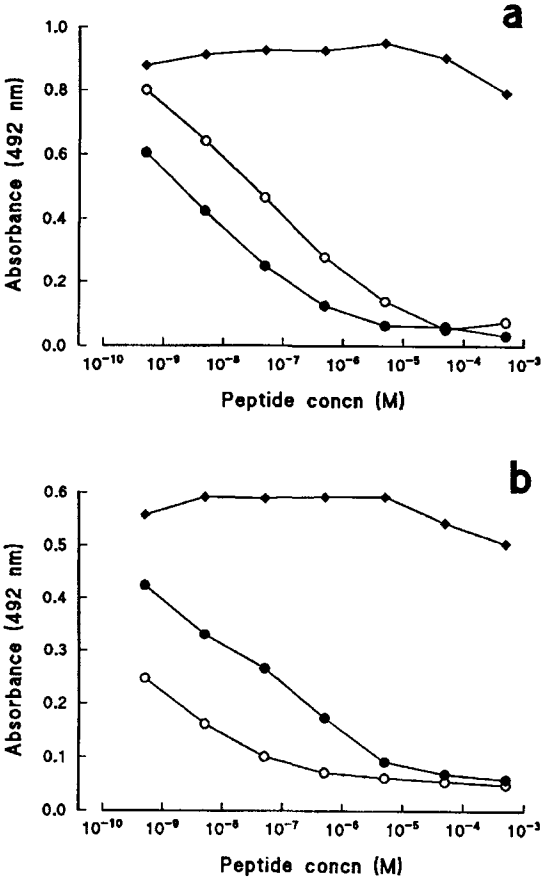

Fig. 1. Competition between peptides and protein antigens for antibody binding. Antiserum against (a) IITGS diluted 1:30000 and (b) VINGA diluted 1:3000, was mixed with a series of concentrations of peptides IITGS (●), VINGA (○), or FSAR (♦). The mixtures were incubated at room temperature for 1 hr and then added to a microtitre plate that had been coated with hepatic microsomal fraction from rats treated with PCN. Antibody binding was determined as described in the text. Each point is the mean of 2 determinations and the data shown are from one experiment typical of three.

To confirm the specificity of binding of the two antibodies to their target protein antigens, each antiserum was incubated with peptide before addition to nitrocellulose filters. In the presence of 0.1 μM or more of IITGS, binding of the anti-IITGS antibody to hepatic microsomal fraction from PCN-treated rats was completely inhibited (Fig. 2a). To inhibit completely antibody binding using VINGA, it was necessary to add 10 μM, although some inhibition was apparent at 1 μM (Fig. 2b). For the anti-VINGA antibody, 0.1 μM VINGA was sufficient to prevent antibody binding to hepatic microsomal fraction (Fig. 2d), whereas 10 μM IITGS was needed to inhibit binding of this antibody (Fig. 2c).

Material immunoadsorbed from solubilised hepatic microsomal fraction from rats treated with PCN by the anti-IITGS antibody was analyzed by immunoblotting. Using the antibody against IITGS (the C-terminus of CYP3A1), a single band corresponding in molecular weight to CYP3A1 was found, whereas the antibody against VINGA (the C-terminus of CYP3A2) was unable to detect any immunoreactive material in this preparation (Fig. 3a). Immobilised anti-VINGA antibody failed to immunoadsorb any antigen (Fig. 3b), possibly due to the lower binding strength of this antibody (Fig. 1b).

Microtitre plates were coated with hepatic microsomal fraction from rats treated with different inducing compounds. The antibody raised against IITGS bound only weakly to hepatic microsomal fraction from UT (or vehicle-treated) rats, but very strongly to hepatic microsomal fraction from rats treated with PCN (Fig. 4a). The antibody showed increased binding to hepatic microsomal fraction from rats treated with STZ compared with that from UT rats. Binding to hepatic microsomal fraction from rats treated with other P450-inducing compounds was similar to that seen in UT rats (Fig. 4a). The antibody raised against VINGA also bound only very weakly to hepatic microsomal fraction from UT rats. This antibody showed a strong increase in binding to hepatic microsomal fraction from rats treated with PCN (Fig. 4b). Binding to hepatic microsomal fraction following treatment of rats with other inducing compounds was in the order PCN > MC > PB > UT = STZ = CFA.

Immunoblotting of hepatic microsomal fractions showed that the antibody raised against IITGS bound to a single band with a molecular weight of 54,000 in UT rats and that the intensity of this band increased markedly following treatment of rats with PCN (Fig. 5a). Quantification studies showed that there was a 250-fold increase in the level of this protein. STZ treatment also caused an increase in the intensity of this band, but treatment with MC or PB reduced the intensity slightly, while treatment with CFA had no effect on the level of this band (Fig. 5a). The antibody raised against VINGA bound to hepatic microsomal fraction from UT rats (Fig.

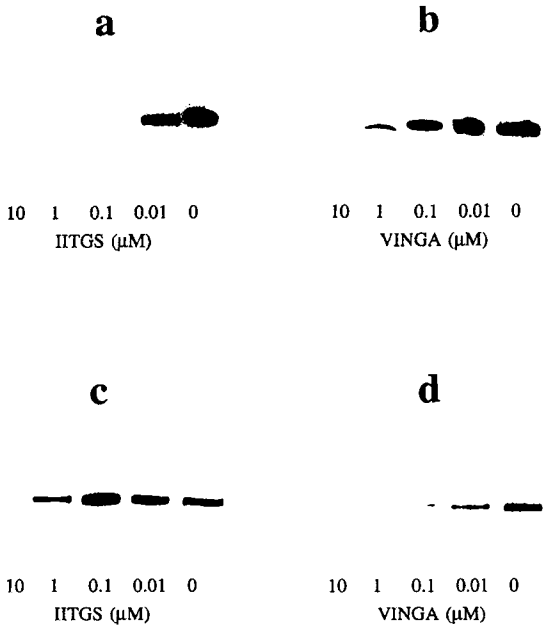

Fig. 2. Inhibition of the binding of antipeptide antibodies to protein antigens by peptides. Immunoblots of 2 μg of hepatic microsomal fraction from rats treated with PCN were developed with either (a and b) anti-IITGS antiserum or (c and d) anti-VINGA antiserum that had first been incubated for 1 hr at room temperature with a series of concentrations of the peptide IITGS (a and c) or VINGA (b and d). Bound antibody was detected as described in the text. Only the central section of each blot containing the immunoreactive bands is shown.

5b). This band migrated to a similar position to that identified using the anti-IITGS antibody. The intensity of the band was increased following treatment of rats with PCN (Fig. 5b). Quantification studies showed that there was a 25-fold increase in the level of this protein. There was a smaller increase in the intensity of this band following treatment of rats with MC or with PB, and a slight increase in STZ- and CFA-treated rats (Fig. 5b).

In hepatic microsomal fraction from UT female rats, the antibody against IITGS bound to a single band and this was strongly induced following treatment with PCN (Fig. 6a). No immunoreactive bands were detected in UT female rats using the anti-VINGA antibody; however, after treatment of rats with PCN, the antibody bound strongly to a single band (Fig. 6b).

In kidney microsomal fraction from UT male rats, the anti-IITGS antibody bound to a single band that migrated to a similar position as the band in liver. There was slight induction of this protein following treatment with PCN but not with any of the other P450-inducing compounds studied (Fig. 7a). Similarly, in microsomal fraction from the lung of UT male rats, the antibody bound to a single immunoreactive band, but this was not induced by PCN, MC or STZ (Fig. 7b). Treatment of rats with PB or CFA reduced the level of this band (Fig. 7b). In the small intestine of UT male rats, a single immunoreactive band of the same molecular weight as that seen in liver was detected and this was induced following treatment of rats with PCN or STZ (Fig. 7c). Treatment with other P450-inducing compounds caused only a slight increase in the intensity of the band. In contrast, the anti-VINGA antibody did not bind to any protein in microsomal fraction from kidney or lung (data not shown), and bound to small intestine microsomal fraction only following treatment of rats with CFA (Fig. 7d). However, the immunoreactive band in the small intestine of rats treated with CFA did not appear to be CYP3A2, because there was a clear difference in the electrophoretic migration of this band and that in the liver (Fig. 7d).

The distribution of CYP3A1 and CYP3A2 was also examined by immunocytochemistry in male rats. In the liver of UT rats, CYP3A1 was found in the centrilobular region (Fig. 8a), although a lower level of staining was also detected throughout the lobule in many, but not all, hepatocytes. After treatment with PCN, there was a very strong even staining in all hepatocytes throughout the lobule (Fig. 8b). CYP3A2 in the liver of UT rats was localised in the centrilobular region (Fig. 8c). Following treatment of rats with PCN, strong immunoreactive staining was found that was concentrated around the centrilobular venules and spread with decreasing intensity into the surrounding hepatocytes (Fig. 8d). In the small intestine of UT rats, only very weak staining of CYP3A1 was observed but, following PCN treatment, there was moderate CYP3A1 immunostaining of villus enterocytes that was stronger at the tips than in the crypts (Fig. 9a and b). In the lung of UT rats, very weak CYP3A1 immunoreactivity was detected in the Clara cells (Fig. 9c) and PCN treatment caused a marked increase of CYP3A1 in these cells (Fig. 9d). Finally, in the

Fig. 3. Immunoadsorption of anti-IITGS antigen in solubilised hepatic microsomal fraction from rats treated with PCN. Solubilised microsomal fraction was incubated with immobilised antibodies and components that bound to the antibody were prepared as described in the text. Immunoblotting was performed using 1 μg of unfractionated solubilised microsomal fraction (lane 1), and 2 μL of supernatants from anti-FSAR-IgG-Sepharose (lane 2), anti-IITGS-IgG-Sepharose (lane 3), and anti-VINGA-IgG-Sepharose (lane 4) and developed with either (a) anti-IITGS antiserum or (b) anti-VINGA antiserum as described in the text.

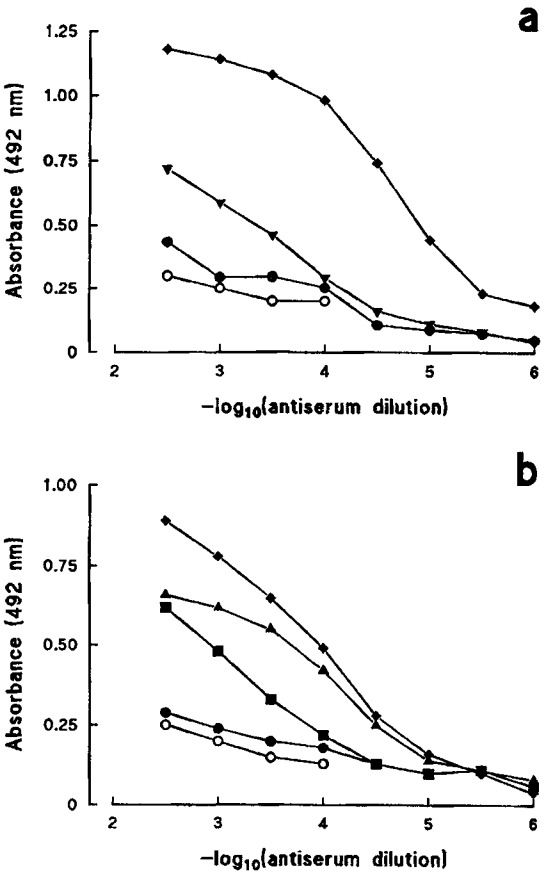

Fig. 4. Binding of antipeptide antibodies to hepatic microsomal fractions from male rats treated with various P450-inducing compounds. Microtitre plates were coated with hepatic microsomal fraction from rats treated with PCN (♦), STZ (▼), MC (▲), PB (■), or vehicle only, or UT rats (●). A series of dilutions of (a) anti-IITGS and (b) anti-VINGA antiserum was added and antibody binding determined as described in the text. Where no result is shown for an inducing compound, the results were indistinguishable from those obtained using UT rat hepatic microsomal fraction. The binding of antibodies in preimmune serum is also shown (○). Each point is the mean of 2 determinations and the data shown are from one experiment typical of the results of three such experiments.

kidney of UT rats, there was weak CYP3A1 immunoreactivity in proximal renal tubules and this staining was increased following PCN treatment (Fig. 9e and f). CYP3A2 was not detected in the small intestine, lung, or kidney of UT rats or rats treated with any of the P450-inducing compounds, except for the small intestine of rats treated with CFA. Although the protein recognised by the antibody in this tissue appears to be other than CYP3A2, immunoreactivity was still localised to the villus (results not shown).

For both antibodies, an absorption test was performed in which antiserum was mixed with peptide before application to the tissue section. For the anti-IITGS antibody, 0.25 μM IITGS prevented the antibody from binding to liver from PCN-treated rats; the same concentration of VINGA had no effect. Conversely, anti-VINGA was competed by 0.25 μM VINGA, while 0.25 μM IITGS had no effect on its binding. In addition, controls in which antiserum was replaced with the same concentration of preimmune serum resulted in no immunoreactivity in any of the sections.

DISCUSSION

We have previously shown that specific antibodies against different forms of cytochrome P450 can be reliably produced by using peptides corresponding to the five C-terminal residues of each P450 as haptens [14]. Although CYP3A1 and CYP3A2 share 89% amino acid sequence similarity, they differ by 3 out of 5 of their C-terminal residues. We, therefore, synthesised peptides corresponding to the five C-terminal residues of these two enzymes in an effort to produce antibodies specific to each form. The results of the present study show that the antibodies, against the synthetic peptides IITGS (the C-terminus of CYP3A1) and VINGA (the C-terminus of CYP3A2) bind strongly to hepatic microsomal fraction from rats treated with PCN, in both ELISA and immunoblotting. In both assays, binding of the anti-IITGS antibody was strongly inhibited by incubation with the peptide IITGS. Incubation with the peptide VINGA was about 100 times less effective at preventing binding,

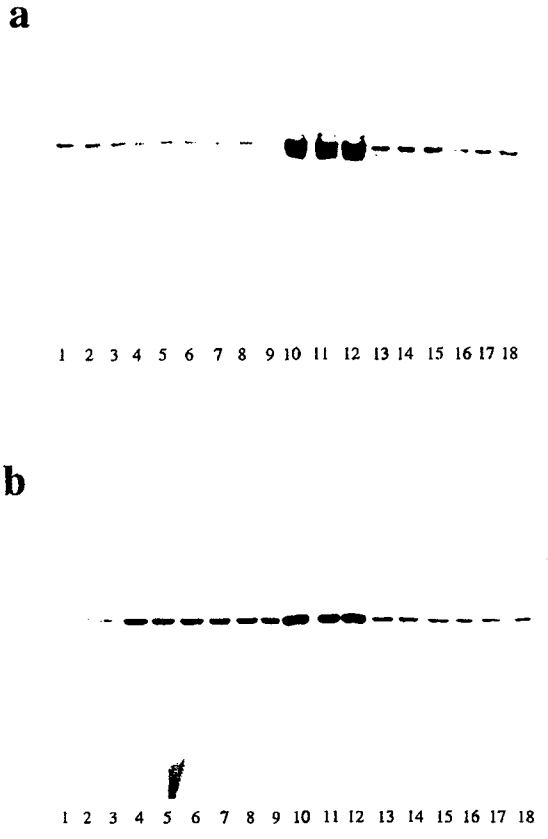

Fig. 5. Immunoblots showing the binding of antipeptide antibodies to hepatic microsomal fraction from male rats treated with various P450-inducing compounds. Samples of 5 μg of hepatic microsomal fraction was applied to each lane as follows: lanes 1–3, UT; lanes 4–6, MC-treated; lanes 7–9, PB-treated; lanes 10–12, PCN-treated; lanes 13–15, STZ-treated; and lanes 16–18, CFA-treated. The immunoblot was developed with either (a) anti-IITGS antiserum, or (b) anti-VINGA antiserum, as described in the text.

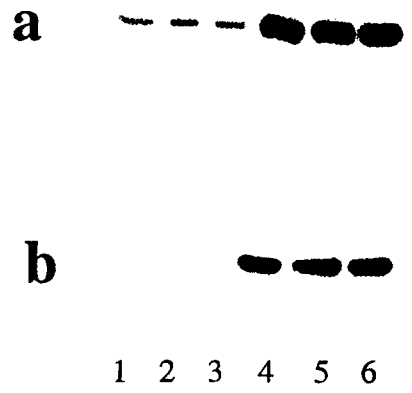

Fig. 6. Binding of antipeptide antibodies to hepatic microsomal fraction from female rats. Samples of 10 μg hepatic microsomal fraction were applied as follows: UT female rats (lanes 1–3), PCN-treated female rats (lanes 4–6). The immunoblot was developed with either (a) anti-IITGS antiserum or (b) anti-VINGA antiserum, as described in the text. Only the central section of each blot containing the immunoreactive bands is shown.

although a structurally unrelated peptide had almost no effect on antibody binding. The anti-VINGA antibody behaved similarly, the homologous peptide VINGA inhibiting binding about 100 times more effectively than the peptide IITGS and, again, the structurally unrelated peptide having no effect. Thus, although the two immunising peptides are both structurally and immunologically related, they are sufficiently different to allow specific binding of antibodies raised against them. Hence, at an appropriate dilution the anti-IITGS antibody appears to bind to CYP3A1 but not CYP3A2, whereas the opposite is true for the anti-VINGA antibody.

The pattern of binding of the two antibodies to hepatic and extrahepatic microsomal fractions from uninduced rats and from animals treated with various inducing compounds shows differences in the expression of proteins recognised by the two antibodies, providing further evidence of antibody specificity. In particular, although both antibodies bound to proteins in the liver that were increased following treatment with PCN, only the anti-IITGS antibody recognised protein in the lung, kidney, and small intestine of UT and PCN-treated male rats, whereas the anti-VINGA antibody failed to recognise any protein in these samples. This indicates that the anti-VINGA antibody binds to a PCN-inducible P450 in liver (i.e., CYP3A2), but does not bind to the same protein to which the anti-IITGS antibody binds (i.e. CYP3A1), which is inducible in extrahepatic tissues. Further, immunoadsorption of solubilised hepatic microsomal fraction from rats treated with PCN using the anti-IITGS antibody yielded just a single protein, which was recognised only by the anti-IITGS antibody. Thus, the binding of the anti-IITGS and anti-VINGA antibodies is mutually exclusive and consistent with specific binding to their target antigens, CYP3A1 and CYP3A2, respectively.

The present study shows that in UT rats CYP3A1 is expressed in the liver of both male and female rats at similar levels. In contrast, a study using specific monoclonal antibodies by Cooper *et al.* [20] failed to detect any CYP3A1 in the liver of male rats. However, this was almost certainly a consequence of the lower affinity of their antibody compared to that used here. In their study, Cooper *et al.* [20] found 330 pmol/mg protein of CYP3A1 in the liver of PCN-treated animals, with a limit of detection of 10 pmol/mg. However, the present study shows that treatment of male rats with PCN results in a 250-fold increase in hepatic CYP3A1 content, thus demonstrating that the constitutive level of expression is well below that detectable by the antibody of Cooper *et al.* [20]. This is an important point, as the current study shows, through the use of more sensitive antibodies, that there appears to be a qualitative difference in the constitutive expression of CYP3A1 and CYP3A2 in the female rat, only the former being expressed. In contrast to CYP3A1, the present study shows that CYP3A2 is expressed in the liver of only male rats, as found in previous studies [20, 21].

It has long been known that CYP3A enzymes are inducible in the rat by PCN. The present study confirms and extends observations on the effects of inducers on the expression of CYP3A1 and CYP3A2 in rat liver. In general, the results of ELISA and immunoblotting were in good agreement. Treatment with PCN causes a very marked increase in CYP3A1 and CYP3A2 content of liver of both adult male and female rats. It is not clear why the monoclonal antibody used by Cooper *et al.* [20] failed to detect any CYP3A2 in PCN-treated females, as this antibody did detect CYP3A2 in the liver of UT males, where expression is certainly no greater than in PCN-treated females. There may be strain differences in the response to PCN; Cooper *et al.* [20] used Long-Evans rats and Wistar rats were used in the present study. There are also age-related changes in the expression and inducibility of CYP3A enzymes, and there were slight differences in the ages of the animals used in the

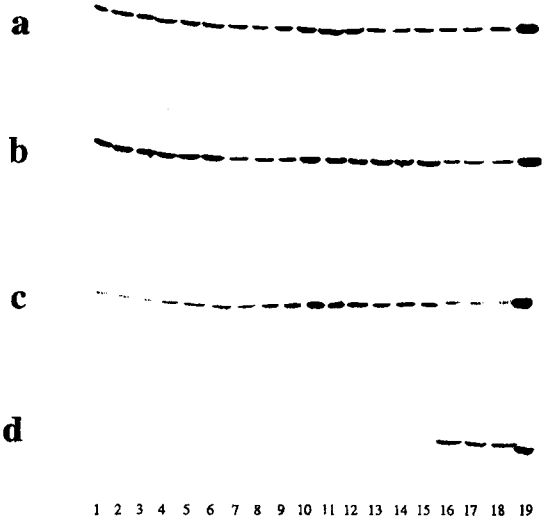

Fig. 7. Immunoblots showing the binding of antipeptide antibodies to extrahepatic microsomal fractions from male rats treated with various P450-inducing compounds. Microsomal fractions were applied to each lane as follows: lanes 1–3, UT; lanes 4–6, MC-treated; lanes 7–9, PB-treated; lanes 10–12, PCN-treated; lanes 13–15, STZ-treated; lanes 16–18, CFA-treated; and lane 19, 1 μg of hepatic microsomal fraction from PCN-treated rat. The samples added were (a) kidney, 25 μg; (b) lung, 50 μg; and (c and d) small intestine, 20 μg; and were developed with anti-IITGS antiserum (a–c) or anti-VINGA antiserum (d), as described in the text. Only the central section of each blot containing the immunoreactive bands is shown.

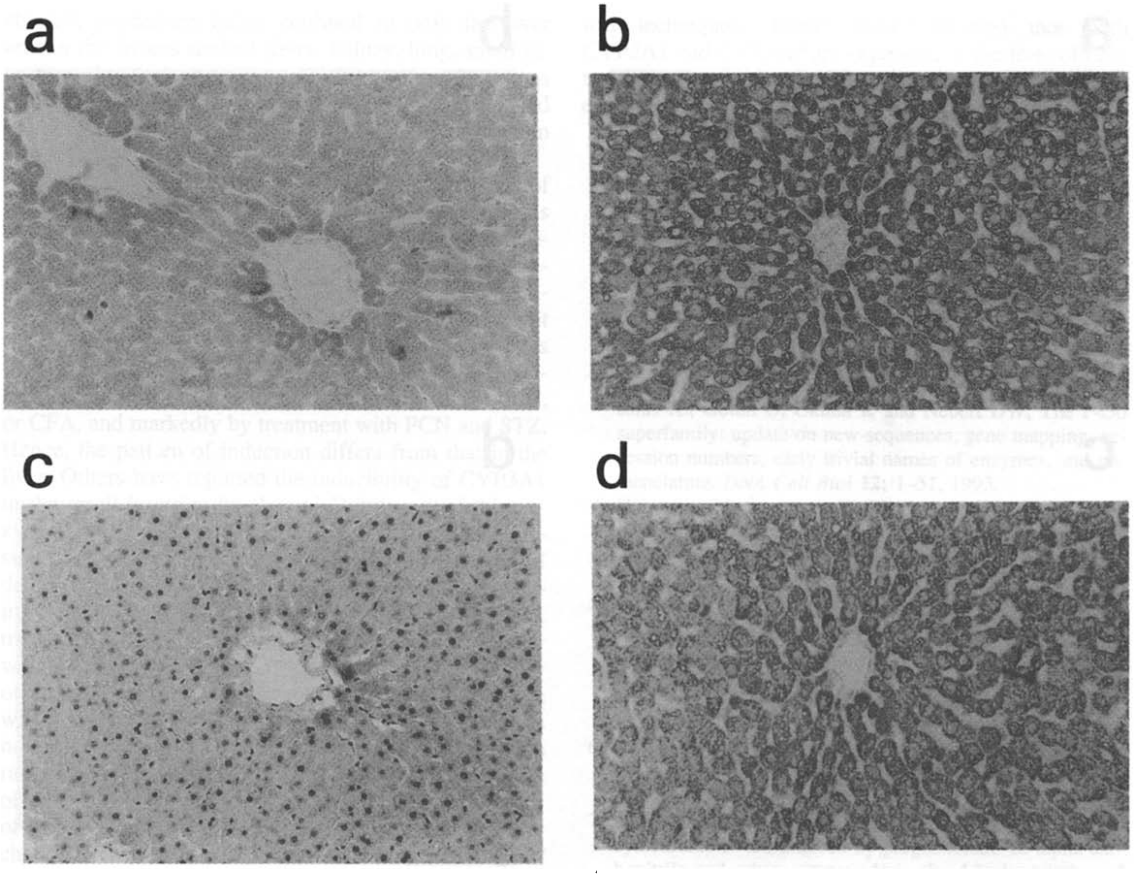

Fig. 8. Immunocytochemical location of CYP3A1 and CYP3A2 in liver of UT and PCN-treated male rats. Sections of liver from UT (a and c) and PCN-treated (b and d) rats were incubated with either anti-IITGS antiserum diluted 1:400 (a and b) or anti-VINGA antiserum diluted 1:100 (c and d), and developed as described in the text to determine the location of CYP3A1 and CYP3A2, respectively. Original magnification is ×200 for all sections.

two studies. An additional possibility is that the various antibodies might bind to other forms of CYP3A not yet fully characterised. It is not clear whether or not forms of CYP3A in addition to the two originally cloned by Gonzalez *et al.* [12, 13], CYP3A1 and CYP3A2, are expressed in rat liver. Certainly, both protein purification [22–24] and cDNA cloning [25] suggest that additional forms may be present. It is not known whether the anti-CYP3A monoclonal antibodies used in other studies would bind to any of these forms; this could only be determined by direct study. In contrast, the possible reactivity of the antipeptide antibodies used in the present study can be predicted as soon as the primary sequences of these forms are known.

PB has been reported to induce CYP3A enzymes, both at the mRNA and protein level, although this does appear to be critically dependent on the age of the animals. In the present study, PB treatment of 14–16-week-old male Wistar rats clearly increased the levels of CYP3A2 and, if anything, causing a decrease in the levels of CYP3A1. Cooper *et al.* [20] reported that PB treatment of Long-Evans rats increased the expression of CYP3A1, determined using a monoclonal antibody. Possible reasons for differences between studies have been discussed above.

Among the other inducers, MC causes marked, but selective, induction of CYP3A2 and, if anything, the levels of CYP3A1 are decreased by this compound. These effects are very similar to those found with PB. Cooper *et al.* [20] also found that MC treatment caused an increase in CYP3A2, whereas the levels of CYP3A1 remained below detection. STZ, a diabetogenic agent in the rat, increased the levels of both CYP3A1 and CYP3A2 to a similar extent. However, the degree of induction is modest, compared with that caused by PCN. Previous studies have shown that other inducers of CYP2E1, such as acetone [26], ethanol, and isopentanol [27], also increase the expression of CYP3A enzymes. CFA, a potent inducer of CYP4A in the rat [28], also induces CYP3A2, but has no effect on the expression of CYP3A1. To our knowledge, this is the first report of CYP3A induction by a member of this class of inducer. CYP3A enzymes are, thus, inducible by a range of inducers that act by different mechanisms. Both MC and CFA normally induce P450 enzymes by activation of their respective receptors, and induction by PCN, PB, and STZ is, in each case, by different mechanisms. It would, thus, be of interest to determine the mechanism of induction of CYP3A by each of these compounds. In several cases there is evidence for increased mRNA levels, although whether this is due to increased transcription or to decreased degradation (mRNA stabilisation),

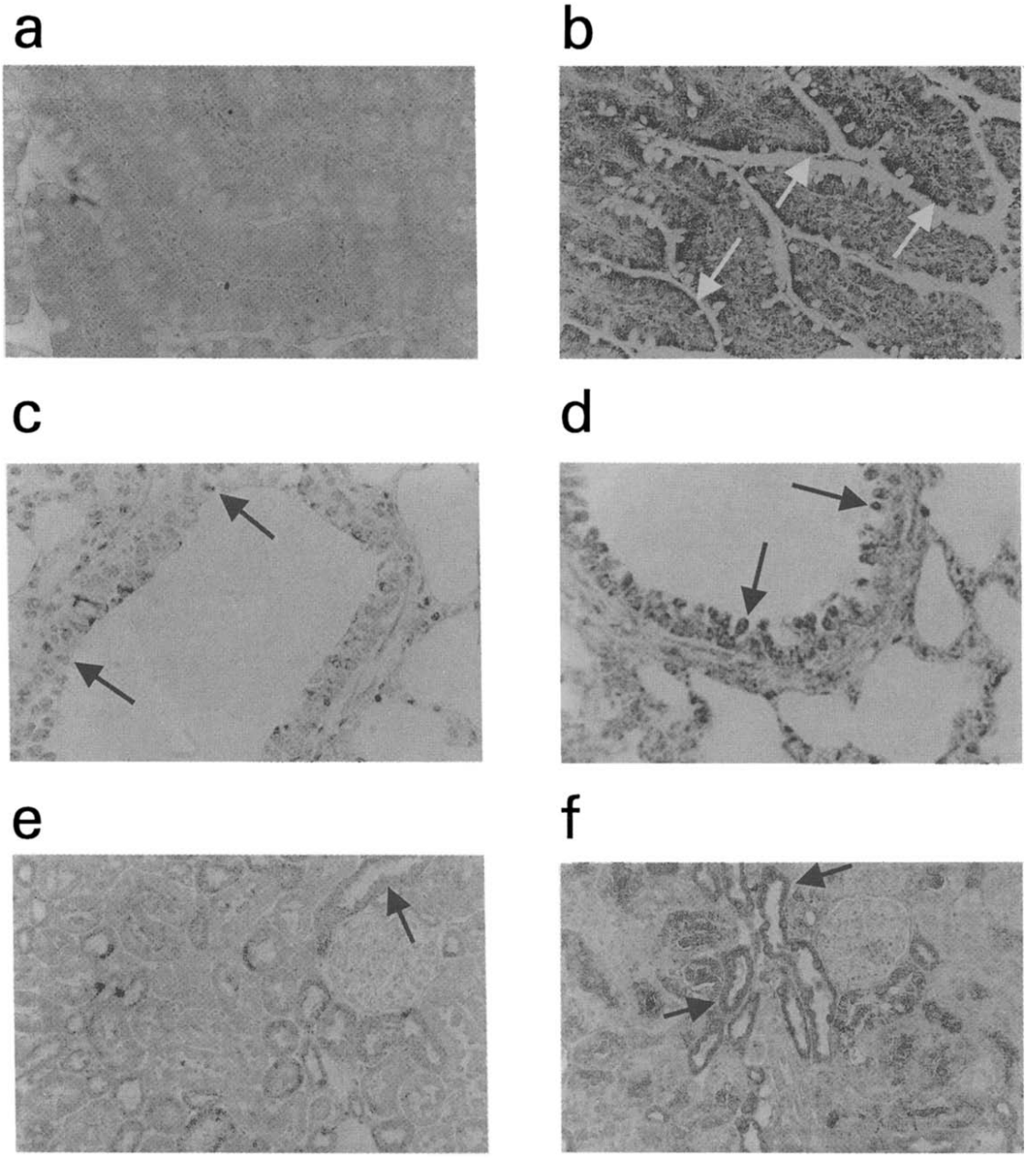

Fig. 9. Immunocytochemical location of CYP3A1 in extrahepatic tissues. Sections of small intestine, lung, and kidney from UT (a, c, and e, respectively) and PCN-treated (b, d, and f, respectively) male rats were incubated with anti-IITGS antiserum diluted 1:200 and developed as described in the text to determine the location of CYP3A1. Arrows indicate immunoreactivity in enterocytes near the tips of villi in the small intestine (b), in the Clara cells of the lung (c and d), and in proximal renal tubules of the kidney (e and f). Original magnification is ×200 for small intestine and kidney sections and ×400 for lung sections.

as has been reported with dexamethasone [29], has yet to be determined.

The hepatic distribution of CYP3A1 and CYP3A2, determined immunocytochemically, differed in both UT and PCN-treated rats. In UT animals, CYP3A1 is expressed in small patches of hepatocytes distributed throughout the lobule, whereas CYP3A2 is expressed mainly in the centrilobular zone. This distribution of CYP3A2 is similar to that reported by Buhler *et al.* [26], using a polyclonal antibody raised against rat P450 PB/PCN-E (CYP3A2) [30]. Following treatment of rats with PCN, CYP3A1 is strongly expressed throughout the lobule and CYP3A2 expression, although increased, is still confined largely to the centrilobular zone (although there is expression in all hepatocytes). Horsman *et al.* [31] found a similar increase in centrilobular staining with a polyclonal anti-CYP3A antibody following dexamethasone treatment, although the intensity and distribution of staining did vary with the dose of inducer.

The tissue distribution of CYP3A2 is remarkably re-

stricted, expression being confined to only the liver among the tissues studied (liver, kidney, lung, small intestine). In marked contrast, CYP3A1 expression has been reported in many tissues, a fact that was confirmed in the present study where expression was observed in all of the tissues examined.

Constitutive expression of CYP3A1 in enterocytes of the small intestine has been widely reported, and this was confirmed in the present investigation by immunocytochemistry. Although low, expression was also apparent by immunoblotting. The enzyme is most abundant at the tips of the villi, with lower levels in the crypt cells. This distribution resembles that reported by others [32, 33]. CYP3A1 content of the small intestine is increased modestly by treatment of animals with PB, MC, or CFA, and markedly by treatment with PCN and STZ. Hence, the pattern of induction differs from that in the liver. Others have reported the inducibility of CYP3A1 in the small intestine by "classic" inducers of this enzyme, such as dexamethasone [33, 34]. CYP3A2 expression in the small intestine remained below the level of detection after treatment of animals with different inducing agents, including PCN. However, following CFA treatment, an immunoreactive band of lower molecular weight than CYP3A2 was strongly induced. The identity of this protein, which was not found in any other tissue with or without treatment of the animals with inducer, is not known. CYP3A1 is constitutively expressed in the rat lung and immunocytochemistry revealed low levels of the protein in several cell types, confirming the work of Voigt *et al.* [35]. Immunoblotting failed to show any change in the levels of CYP3A1 following treatment of the animals with PCN. However, immunocytochemistry revealed that PCN causes a marked increase in the expression of this protein in Clara cells. This cell type has been shown previously to possess high levels of several drug-metabolising enzymes and to be a target for a number of toxins requiring metabolic activation [15, 36–38]. These studies illustrate, again, how potentially misleading measurements can be using subcellular fractions of tissues comprising heterogeneous cell types, such as the lung.

CYP3A1 is readily detectable in microsomal fractions from rat kidney, but the levels of this enzyme did not change following treatment of the animals with any of the inducers studied, other than a very modest increase after PCN. Immunocytochemistry revealed that constitutive expression is confined to the proximal convoluted tubules of the cortex; this is increased considerably by PCN treatment of the animals. Proximal tubular cells have been shown previously to respond to inducers of CYP1A1 [38].

In conclusion, it has been possible to produce specific antibodies against CYP3A1 and CYP3A2 by using the respective C-termini as haptens. The peptides used were only 5 amino acids in length but, despite this small size, the resultant antibodies bound strongly to the target antigens. A difference in 3 out of 5 amino acid residues was sufficient to produce antibodies that could discriminate between the two forms of P450. Not only did these antibodies bind strongly to P450 in ELISA and immunoblotting, they also bound well to their antigens in formaldehyde-fixed sections of tissue. It was, therefore, possible to use the antibodies to study the distribution and inducibility of CYP3A1 and CYP3A2 in liver, lung, small intestine, and kidney using a range of immunological techniques. These studies showed that both CYP3A1 and CYP3A2 are expressed in the liver of control and PCN-treated male rats, whereas only CYP3A1 is expressed, or inducible, in extrahepatic tissues.

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